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Table(s)

Table 1 Composition of degumming solutions before and after filtration

	Degumming solution	
	Before filtration	**After filtration**
Moisture (%)	98.94	97.57
Ash (%)	0.12	0.11
Total Nitrogen (%)	0.11	0.01
Protein (Nx6.25) (%)	0.69	0.06
pH	9.24	7.20
COD (mg/L)	8,870	260
BOD_5 (mg/L)	4,840	158
Total solid (%)	1.06	0.01

Each sample was analysed in triplicate.

Table 2 Amino acid composition in degumming solution compared with reference sericin

Amino acid	% gram amino acid in 100 gram protein		
	Sericin raw material[1/]	**Degumming solution[2/]**	**Hot water-soluble Sericin[3/]**
Aspartic acid	**15.74**	**14.95**	**17.97**
Serine	**31.99**	**38.81**	**28.00**
Glutamic acid	6.28	3.93	6.25
Glycine	14.20	14.45	16.29
Histidine	1.49	Not detected	1.32
Arginine	4.29	3.27	3.52
Threonine	**7.73**	**7.79**	**7.78**
Alanine	4.85	5.13	5.20
Proline	0.71	0.47	Not detected
Cystine	0.20	Not detected	0.69
Tyrosine	3.01	2.44	2.87
Valine	3.30	3.33	3.77
Methionine	Not detected	Not detected	Not detected
Lysine	**4.17**	**3.12**	**3.72**
Isoleucine	0.72	0.77	0.79
Leucine	0.96	1.18	1.21
Phenylalanine	0.37	0.34	0.64

[1/, 2/]from laboratory analysis

[3/]from Zhang et al. (2004)

Table 3 Chemical composition of recovered sericin powder

Composition	Tray-drying	Freeze-drying	After filtration	Sericin hydrolysate	Commercial SERICIN-P[1/]
Moisture (%)	4.73	4.83	5.19	4.96	12.00
Nitrogen (%)	12.18	12.28	14.75	14.89	13.00
Protein (Nx6.25) (%)	76.10	76.73	92.19	93.06	81.25
Ash (%)	36.89	21.68	5.84	5.42	3.00
pH	9.60	9.27	7.85	7.74	Not determined
Water solubility (%)	96.28	97.58	97.62	100.0	Not determined
Yield (%)	0.93	1.04	95.0	91.3	Not determined

[1/] commercially hydrolysis of sericin

Figure(s)

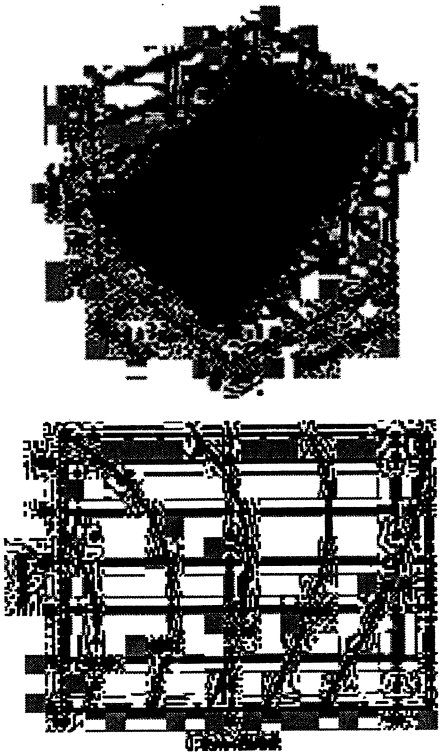

Figure 1 Influence of enzyme concentration and reaction time on the hydrolysis level as shown by Response Surface Curve

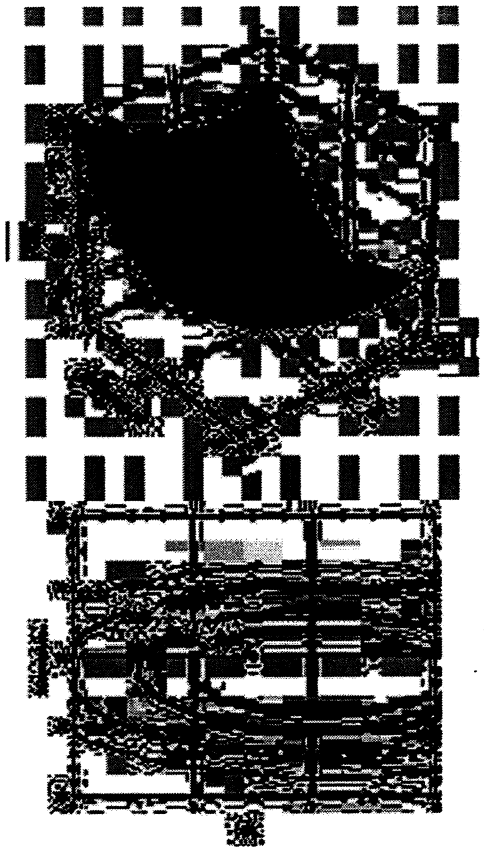

Figure 2 Influence of pH and temperature on the hydrolysis level as shown by Response Surface Curve

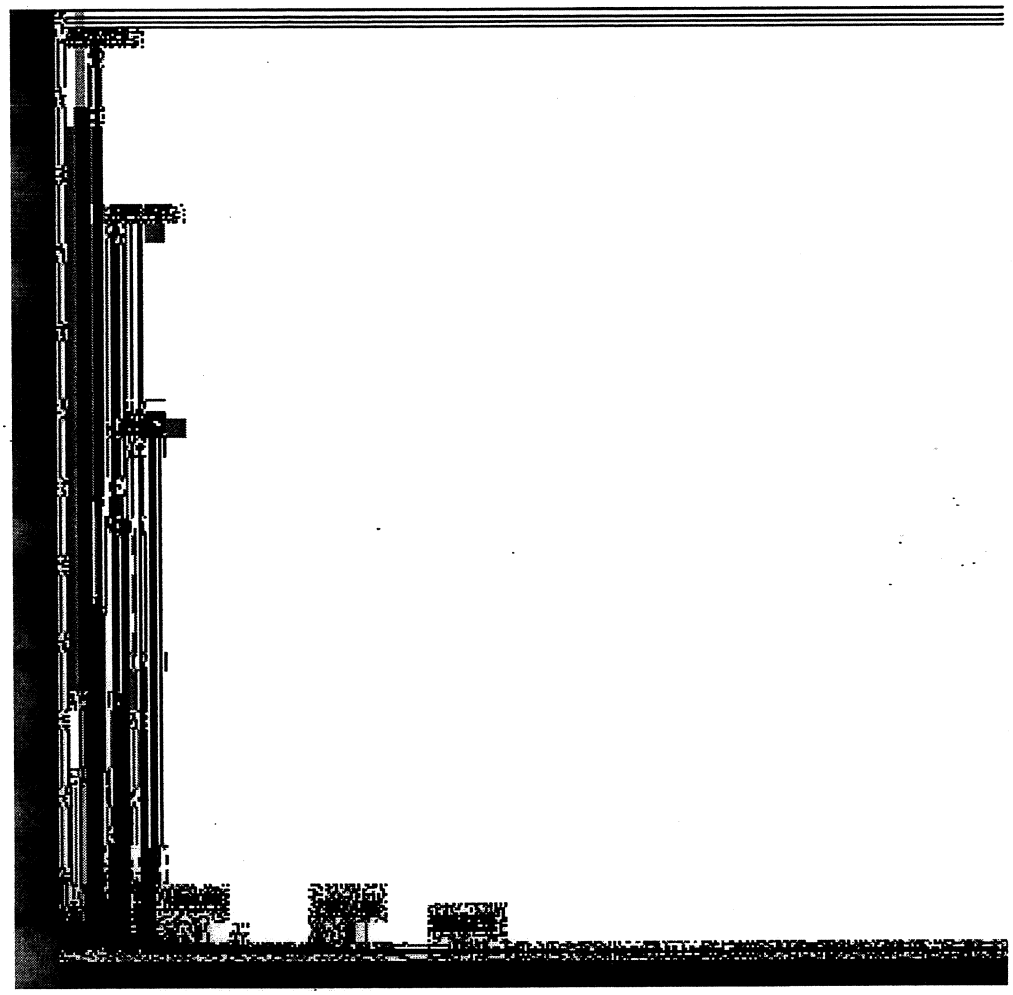

Figure 3 Mass spectrum of sericin powder

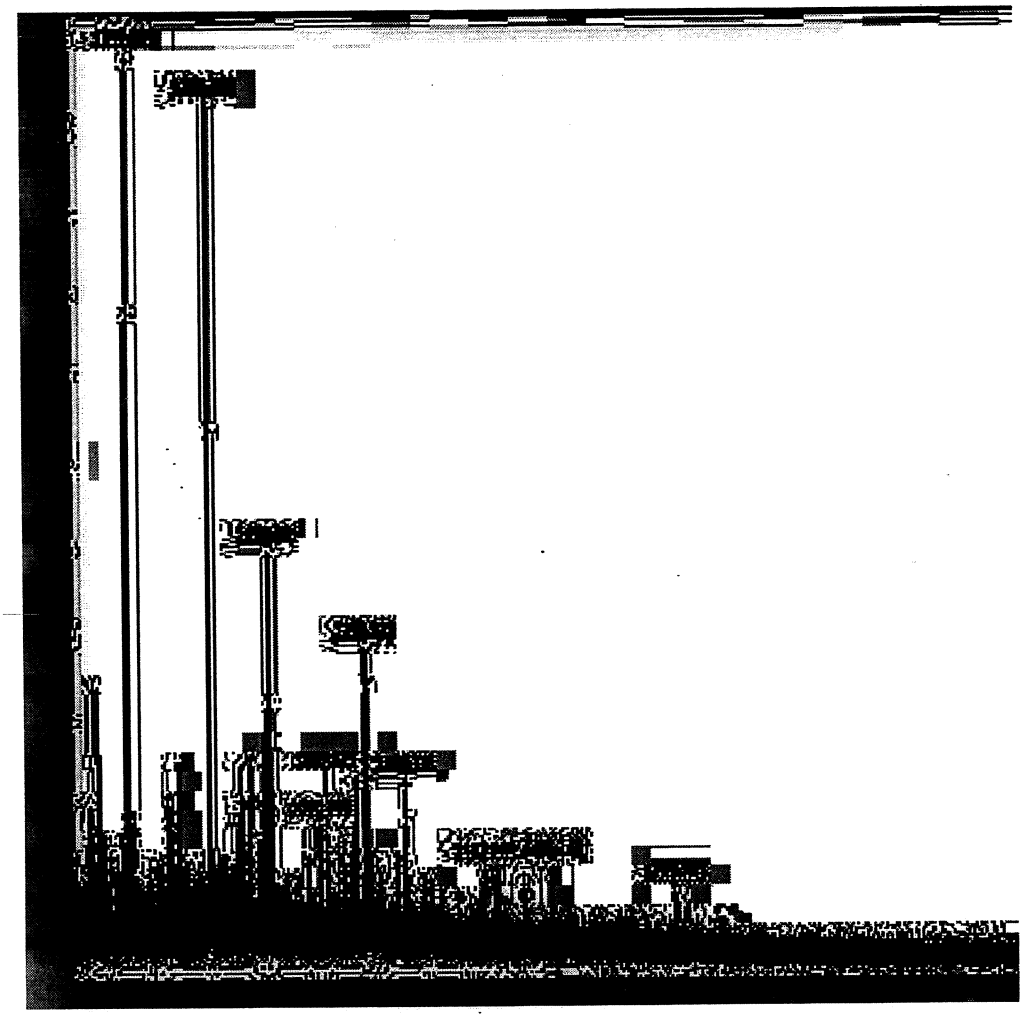

Figure 4 Mass spectrum of sericin hydrolysate

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There was a great response to the call for papers for the upcoming ASPIRE conference. We received nearly 500 abstracts. We had only a limited space for the oral presentations, so we co not include your paper in the oral publication list. This decision does not indicate that your pap of low quality. We have selected oral presentations on the criteria of how the paper would be received by the participants.

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Paper Status:	**Accepted**
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Sericin recovery from silk degumming waste water

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Abstract
This study was conducted to the recovery of sericin protein produced during silk degumming process. Sericin waste solution from conventional degumming process in Thailand contained high BOD (4,840 mg/L), COD (8,870 mg/L) and nitrogen content (0.11%). This indicated protein contamination and highly cost for wastewater treatment. To reduce the treatment costs as well as to recover valuable sericin protein, membrane filtration and enzymatic hydrolysis of recovered sericin were studied. The results showed the quality improvement of wastewater (BOD, 158 mg/L; COD, 260 mg/L) as well as that an amount of sericin protein with molecular weight 2,427-9,863 daltons were recovered after membrane filtration process. The recovered sericin was further enzymatically hydrolyzed to obtain sericin hydrolysate (average molecular weight 1,046-2,795 daltons) which is mostly suitable for further cosmetics application.

Key words
sericin recovery, silk degumming wastewater

INTRODUCTION

Silk manufacturing is one of the industrial sectors where intensive water consumption cannot be avoided therefore resulting in large volume of wastewater. One of these is released from degumming process which is used for elimination external sericin prior to dyeing; generally synthetic soap solution is used at 95 °C for 1 hour. It is expected that one hundred kg of silk produces 22 kg of sericin. Sericin is globular protein representing as a tube outside the silk fibroin with its molecular weight distributed between 10,000 and 300,000 dalton (Fabian *et al.*, 1996; Zhang *et al.*, 2004). When subjecting to alkaline degumming process, sericin is degraded into sericin peptide or hydrolyzed sericin with molecular weight less than 20,000 dalton (Zhang *et al.*, 2004). This peptide and hydrolysate of sericin have excellent moisture absorption property and a lot of biological activities such as antioxidation, tyrosinase activity inhibition and anticancer activity (Chang-Kee *et al.*, 2002; Kato *et al.*, 1998). As a result, they can be applied in many fields such as cosmetics, biomaterials and textile (Zhang, 2002).

High concentration of BOD, COD and nitrogen in degumming waste solution makes it complicated and highly costs for treatment (Rigoni-Stern *et al.*, 1996; Fabiani *et al.*, 1996). Likewise, it also indicated that the high nitrogen content in such wastewater could be sericin-derived products which therefore could be recovered and applied for various purposes. The aim of this work was thus to recover sericin protein as well as to improve the wastewater quality of degumming waste for further applications.

METHODS

The degumming waste solution

Degumming waste solution from *Bombyx mori* silk processing was obtained from silk manufacturer in Thailand. The contents of total nitrogen, protein, moisture and ash of the sample were measured according to AOAC 2000. Total amino acid was analyzed according to AccQ. Tag Amino Acid Analysis Method (1993). Mass spectra were acquired by Bruker Daltonics model reflex IV N_2 laser (337nm). This instrument was operated in linear mode with delay extraction and calibrated with protein standard (Bruker p/n 206195). The spectra were acquired in mass range 1000-100000 amu summing 100 laser shots for each sample. COD and BOD5 of degumming waste solution were analyzed using closed reflux method 5220C and 5210A, respectively, according to APHA-AWWA-WEF (1995).

Protein recovery

A part of degumming waste solution was directly dried using freeze-drying (Labconco Corporation: 77535-01, USA) and tray-drying (Memmert: UM 500, USA). Another part was passed through ultra-filtration (Amicon model 8400, Amicon Inc., USA) with membrane (molecular weight cut-off in the 20,000-80,000 dalton range, Millipore Co., USA) in order to obtain the concentrated sericin protein. After that, the recovered sericin was freeze-dried. Every product derived from the above-mentioned methods were ground into fine powder and then analyzed for contents of total nitrogen, protein, amino acids, moisture and ash according to the previous section and compared with the commercial sericin.

Enzymatic hydrolysis of recovered sericin protein

Dry sericin protein from previous section was subjected to enzymatically (Alcalase 2.4 L FG, Novozymes A/S, Denmark) hydrolysis for a period of time to obtain short-chain peptides or sericin hydrolysate. The pH change during the reaction was controlled by the addition of 0.1 M NaOH. Then the hydrolysate was freeze-dried and analyzed for properties according to the previous section. The hydrolysis parameters, including pH, temperature, reaction time and enzyme concentration, were optimized using Completely Randomize Design (CRD). Data analysis was performed using the Response Surface Methodology (RSM). Following the above-mentioned production process for enzymatic hydrolysis but using 3 different pH (7.5, 8.5, 9.5), 3 different temperatures (50, 55, 60 °C), 4 different reaction times (60, 90, 120, 150 min) and 3 different enzyme concentrations (0.1, 0.5, 1.0% (w/w, mg-enzyme/mg-substrate)). The pH-stat technique was used for monitoring degree of hydrolysis (%DH). The conditions with 95% confidential level ($p \leq 0.05$) and $R^2 \geq 0.75$ were selected for contour plot and the most optimum hydrolysis condition was chosen.

RESULTS AND DISCUSSION

The degumming waste solution

The composition of sericin waste solution was shown in Table 1. It demonstrated high moisture as 98.94% whereas the protein and ash contents were 0.69% and 0.12%, respectively. These were consistent with total solid content as 1.06%. Also sericin yields obtained after freeze-drying and tray-drying were only 1.04% and 0.93%, respectively (Table 3). Total amino acids in sericin raw material, degumming waste solution, hot-water soluble sericin and fibroin raw material were compared in Table 2. It was shown that the majority of amino acids in degumming waste solution were serine, aspartic acid and glycine as 38.81%, 14.95% and 14.45%, respectively, which was similar to those in sericin raw material; serine 31.99%, aspartic acid 15.74% and glycine 14.20%; and hot water-soluble sericin; serine 28.00%, aspartic acid 17.97% and glycine 16.29% (Zhang *et al.*, 2004). In contrast, different amino acids profile was found when compared with those in fibroin; serine 13.22%, aspartic acid 0.76% and glycine 41.25% (Hemachantorn, 1996).

Furthermore, analysis of degumming waste solution indicated high BOD and COD, 4,840 mg/L and 887 mg/L, respectively. Rigoni-Stern *et al.* (1996) and Fabiani *et al.* (1996) also reported similar results due to high nitrogen residues and therefore treatment is needed for this kind of waste water before discharge.

Sericin recovery by drying and filtration

The results in Table 3 showed proximate compositions of sericin powder after freeze- and tray-drying compared to the commercially available sericin. It indicated that both drying methods could unswervingly make degumming waste solution dried and yielded sericin powders with similar compositions except their water solubility. The 10-g freeze-dried sericin was completely solubilized in 100-ml water whereas an equal amount of tray-dried sericin was dissolved in 100-ml water at 96.28%. This could be because the heating process denatured protein during the process as observed by occurring of thin film on the top and after that when tested for water solubility, only this film was not water-soluble. Therefore, freeze-drying was selected for drying degumming waste solution in the next experiment.

Even though sericin could be easily directly recovered from degumming waste solution by drying, the results from Table 3 demonstrated very high ash content in sericin products, 21.68% and 36.89% from freeze- and tray-drying, respectively. High ash contents were from alkali solution used in silk degumming process. Moreover, Table 3 also showed rather low total solid content as little as 1.06% indicating that almost 99% of the degumming waste solution was water and then high energy consumption was needed for drying process. This suggested that removal of alkali ions from degumming solution and reduction of solution volume prior to drying process were extremely essential.

Table 1 showed the composition of degumming waste after being passed through the ultrafiltration system. It demonstrated the obvious decrease in pH, COD, BOD_5 and total solid of the solution from 9.24 to 7.20, 8,870 to 260 mg/L, 4,840 to 158 mg/L and 1.06 to 0.01%, respectively, indicating the quality improvement of degumming waste. Moreover, the decrease in total nitrogen and protein contents, from 0.11 to 0.01% and from 0.69 to 0.06%, respectively, implied the recovery of protein from waste solution. The results illustrated that ultrafiltration with membrane (20,000-30,000 dalton cut-off) of degumming waste allowed the recovery of sericin, with molecular weight 2,427-9,863 daltons, of about 94%. Comparison of recovered sericin as shown in Table 3 indicated better quality, as well as the yield, of sericin obtained after membrane filtration. The protein powder contained higher nitrogen content (14.75%) with lower ash content (5.84%) and pH (7.85) when compared with the tray-dried powder (12.18%, 36.89% and 9.60, respectively) and freeze-dried powder (12.28%, 21.68% and 9.27, respectively). This indicated that removal of impurities by membrane filtration could actually improve the quality and yield of sericin.

Enzymatically hydrolysis of recovered sericin

Alcalase, a commercial serine protease from *Bacillus licheniformis*, was used in this study. This enzyme was experimented by Park *et al.* (2002) as the most efficient protease among the industrially-applicable enzymes for silk fiber hydrolysis. DH values from every hydrolysis condition were determined and compared. The highest DH, about 83.78%, was obtained from the most optimum hydrolysis condition as shown in Figures 1 and 2. Figure 1 showed that the rate of reaction measured by degree of hydrolysis depended severely on enzyme concentration ($p \leq 0.05$). As the enzyme concentration rised, the hydrolysis level was consistently increased. This indicated that addition of enzyme was a decisive factor affecting the hydrolysis rate and it could be small or even unnecessary in the case of raw materials containing active endogeneous enzymes such as wastes from fish or chicken (Kristinsson and Rasco, 2000). Figure 1 also suggested that the rate of

reaction was slightly dependent on reaction time, therefore the shortest duration was chosen according to the lowest energy consumption. These data then allowed us to conclude that the optimum enzyme concentration and reaction time for sericin hydrolysis was at 1% (w/w, mg-enzyme/mg-substrate) and 60 min, respectively.

Figure 2 illustrated the influence of pH and temperature on the protein hydrolysis level. The reaction temperature slightly affected the hydrolysis level as the optimal temperature for this enzyme activity fell in 55-60 °C according to the instuctions. However, the reaction rate was strongly dependent on pH as it affected the ionization of protopic moiety in the enzyme active site, therefore resulting in the activation or inactivation of enzyme activity. Usually, each enzyme has its own optimal reaction pH. The enzyme used in this study is efficiently active in pH range of 8.0-9.5, therefore resulting in best hydrolysis in that range. From the above observations, it was concluded that the optimum pH and temperature for sericin hydrolysis was at 9.5 and 60 °C, respectively. Therefore, reaction pH adjustment is needed prior to carry out the process.

Average molecular weight of sericin powder was analysed. Figures 3 and 4 demonstrated mass spectrums of sericin protein in degumming waste water and sericin hydrolysate after enzymatic hydrolysis, respectively. These mass spectrums indicated molecular sizes of peptides in Daltons unit present in the samples. From figure 3, it was shown that the average size of peptides before hydrolysis ranged between 2427 and 9863 Daltons whereas the 1046-2795 Dalton peptides were observed after enzymatic hydrolysis (Figure 4).

CONCLUSION

The protein powder, containing similar amino acids profile with those in sericin, could be directly recovered from silk degumming waste solution by ways of drying or ultrafiltration. However, the better quality of sericin with higher recovery percentage was obtained from the ultrafiltration method. Also this method provided the improvement of degumming waste solution with lower pH, COD, BOD_5 and total solid indicating as the proper method to recover valuable protein as well as to wastewater treatment. Further hydrolysis with specific commercial protease provided sericin hydrolysate with molecular weight range between 1,046 and 2,795 daltons. Following these results, the membrane filtration and enzymatic hydrolysis show tempting perspectives for management and value-adding of the silk degumming waste. However, the field test as well as cost-benefit evaluation are needed for confirmation and scale-up.

ACKNOWLEDGEMENTS

The authors would like to thank Thailand Research Fund (TRF) for financial support and Chul Thai Silk Co., Ltd. for silk degumming waste water throughout the research.

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Threonine	**7.73**	**7.79**	**7.78**	**0.81**
Alanine	4.85	5.13	5.20	28.87
Proline	0.71	0.47	Not detected	Not detected
Cystine	0.20	Not detected	0.69	Not detected
Tyrosine	3.01	2.44	2.87	10.96
Valine	3.30	3.33	3.77	2.63
Methionine	Not detected	Not detected	Not detected	Not detected
Lysine	**4.17**	**3.12**	**3.72**	**0.17**
Isoleucine	0.72	0.77	0.79	0.44
Leucine	0.96	1.18	1.21	0.32
Phenylalanine	0.37	0.34	0.64	0.58

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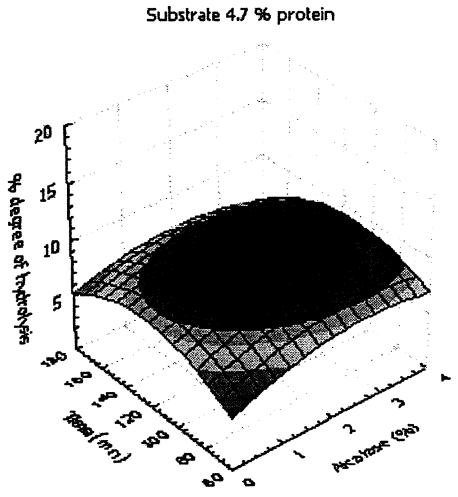
Protein (Nx6.25) (%)	76.10	76.73	92.19	93.06	81.25
Ash (%)	36.89	21.68	5.84	5.42	3.00
pH	9.60	9.27	7.85	7.74	Not determined
Water solubility (%)	96.28	97.58	97.62	100.0	Not determined
Yield (%)	0.93	1.04	95.0	91.3	Not determined

[1/] commercially hydrolysis of sericin



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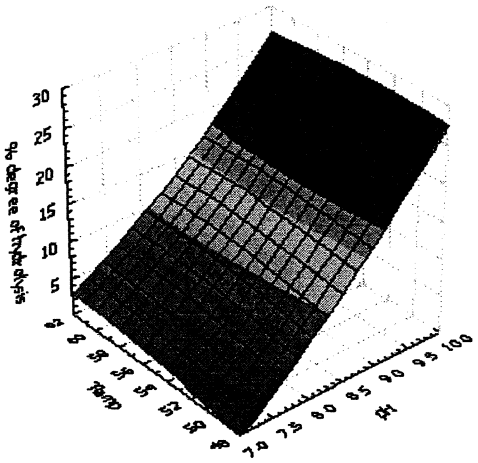
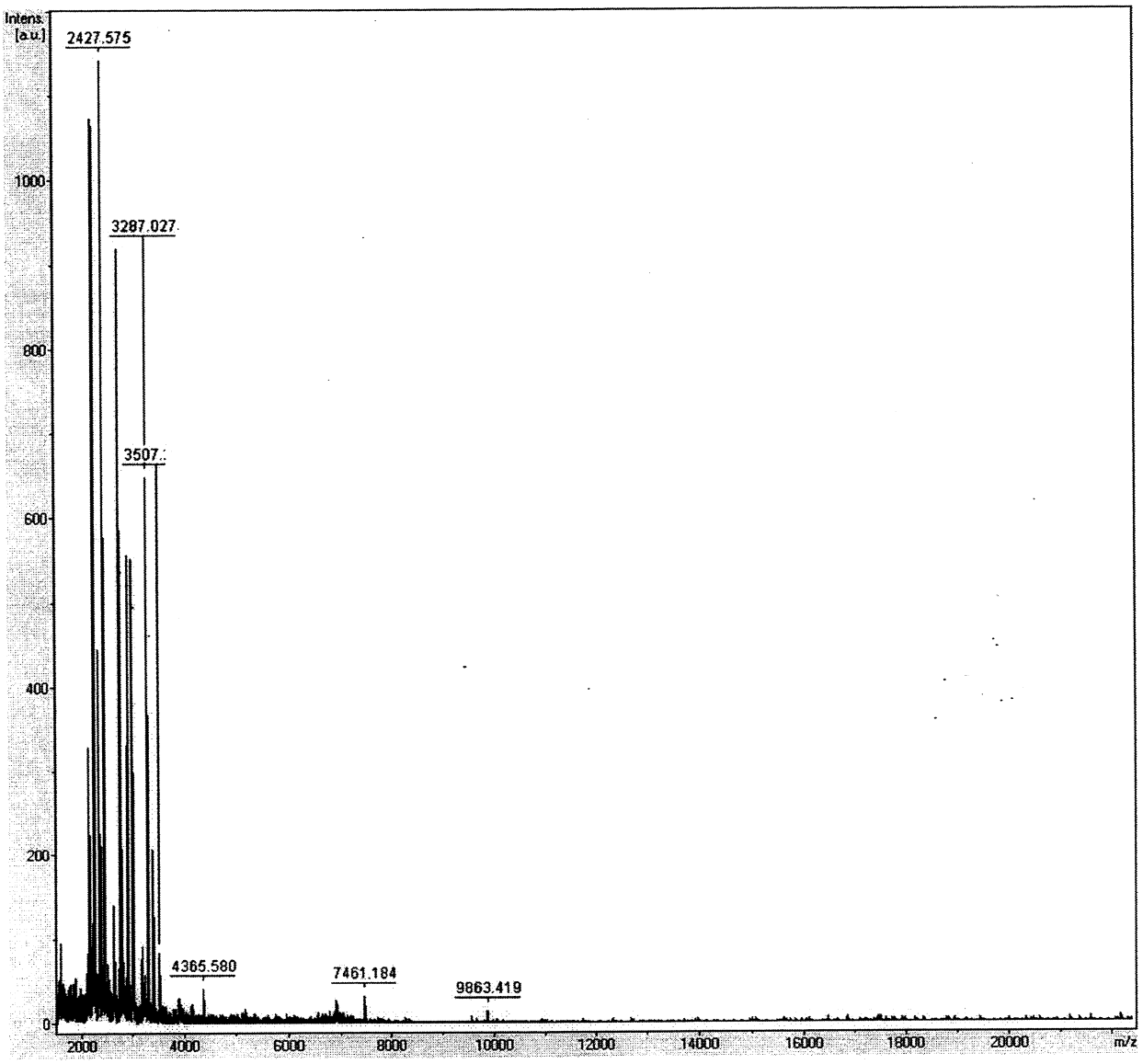


Figure 2. Influence of pH and temperature on the hydrolysis level as shown by Response Surface Curve



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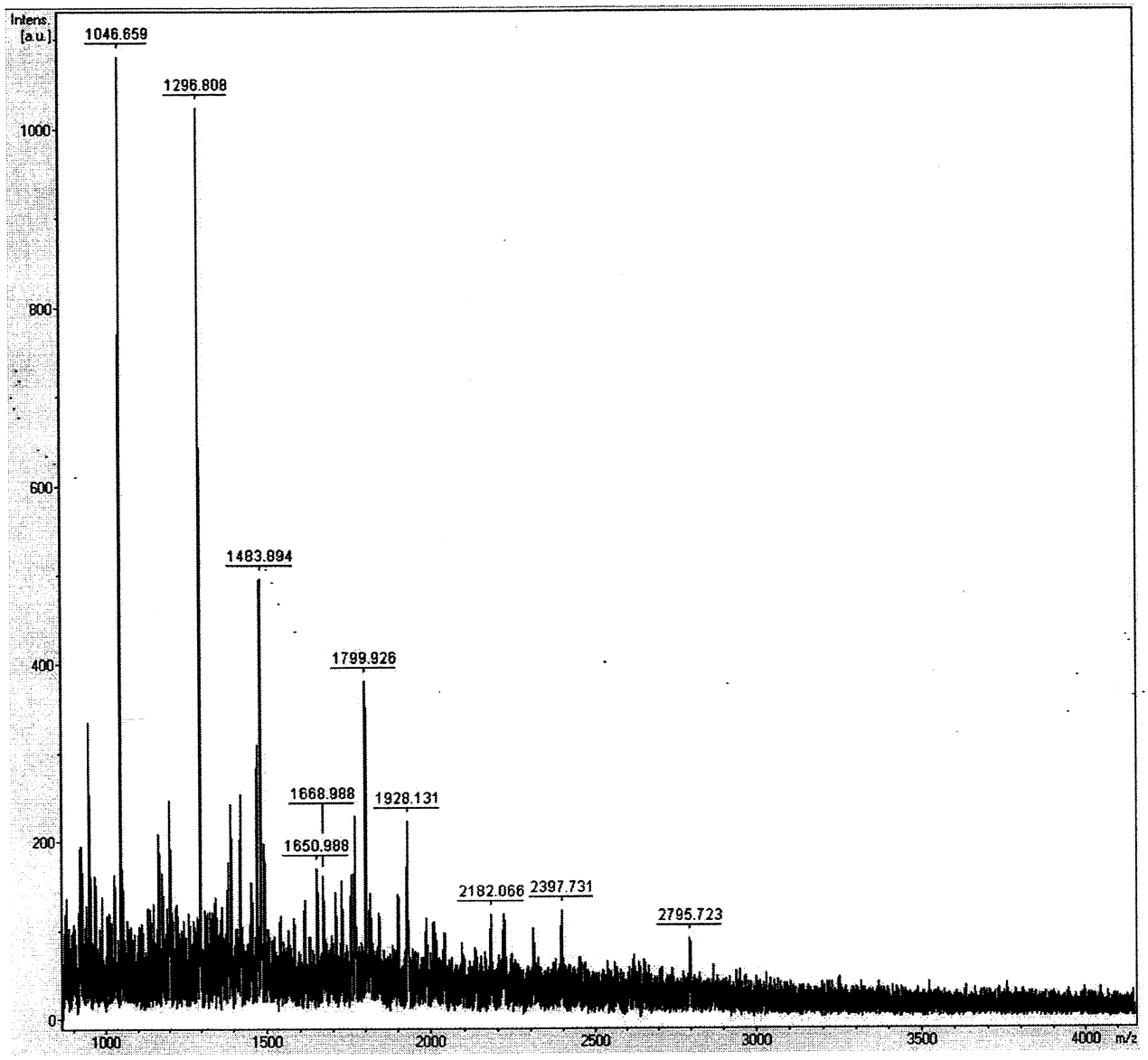

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Dear Dr. Pilanee,

Thank you very much for submitting the manuscript entitled Optimization of the Production Process for Silk Hydrolysate from *Philosamia ricini* (Iri Silk) Pupae (Code 0609-756, received 05 September 2006), by Pilanee Vaithanomsat and Chidchai Punyasawon, for consideration for publication in ScienceAsia.

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Process Optimization for the Production of *Philosamia ricini* (Eri Silk) Pupae Hydrolysate

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ABSTRACT

The method for protein hydrolysate production from Eri silk pupae (*Philosamia ricini*), waste from silk reeling process, was investigated. The appropriate process started by blending fresh pupa into fine particles and water was added to adjust final protein concentration to 4.6 %. The pH was adjusted to 7.5 to accommodate the enzyme activity. Commercial enzyme Alcalase was added to 0.5 % and the process was carried out at 50 °C for 120 min. with stirring. The protein hydrolysate from silk pupa was freeze-dried, ground into fine powder and analysed for compositions. The maximum degree of hydrolysis resulted from this condition was 73.27 % with nitrogen recovery 62.82 %. The results indicated that the hydrolysate product was capable of well dissolving in water and were as high quality as the commercial products.

Key Words: silk hydrolysate, Eri silk pupae, production, optimization

INTRODUCTION

In recent years, natural products have grown from a niche segment to one of the fastest-growing categories in personal care with market value 2,000 million US dollars

per year[1]. Cosmetics are the key products, and ingredients with specific properties are extremely needed. Silk protein or silk protein hydrolysate are one of such ingredients, which can improve skin look and cover skin problem[2,3,4,5]. Especially the hydrolyzed silk contains silk peptide with average molecular weight 1,000 daltons which are water-soluble and well compatible with surfactant resulting in fibroin film that is shining and could be easily coated onto human hair and skin.

The by-products of manufacturing silk include the unusable parts of the pupa and cocoon. Cocoon can be processed to make Dupion silk, or re-processed into flow-silk and spun-silk yarns. Parts of it could be chemically dissolved using LiBr aqueous solution and further processed to obtain silk fibroin membrane as immobilization matrix[6,7]. The pupae can be sold for fertilizer, biogas[8], feed stuff[9,10] and other agricultural purposes[1]. Furthermore, Yang (2002) also reported that silkworm pupas have been used as Chinese traditional medicines since ancient time[11]. Pharmacological studies show that silkworm pupas are alimental for increasing immunity, protecting the liver and preventing cancer. Proximate analysis of pupa showed that it contains 55–60% protein, 25–30% lipid, 4.96% fiber, and other substances, e.g. hormones, trace elements and vitamins, thus indicating that it could be a good protein source for various purposes[10,11] One approach to upgrade silk pupa by-products was use of proteases by choosing the enzyme type and hydrolysis conditions to obtain the hydrolysed silk pupa protein product. Therefore, the main purpose of this research was to optimize the enzymatic hydrolysis condition suitable for silk pupa protein hydrolysate which could be further applied for various products.

MATERIALS AND METHODS

Preparation and analysis of raw material and products

The *Philosamia ricini* (Eri Silk) pupa was used in this study. It was ground into fine particles using Vita mix® and then stored at temperature -20 °C until use. Raw material and all products were sampled for proximate analysis; moisture, ash, protein, lipid and microbiological properties as described in the AOAC (2000)[12]. The molecular weight patterns of hydrolysate were accessed by 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie staining (Pharmacia: EPS 300).

Enzymatic hydrolysis of silk pupae

The production of a hydrolysate was performed as follows (adapted from Chang-Kee *et al.*, 2002)[4]: ground silk pupae were mixed with water to obtain optimal protein content with final volume 100 g. Before subjecting to enzymatically hydrolysis, the suspension was adjusted to suitable pH with 4 N NaOH. The reaction mixture was incubated at appropriate temperature with continuous stirring for a period of time. The pH of reaction was controlled by addition of 4 N NaOH whose volume was recorded for calculation of %degree of hydrolysis (%DH). %DH is defined as the percentage of peptide bonds cleaved during the hydrolysis reaction[13].

$$\%\,DH = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times 100$$

wherein B = Volume of NaOH (ml) used in hydrolysis reaction

N_b = NaOH concentration (N)

MP = Protein content (N x 6.25) (gram)

α = Degree of dissociation of the α-NH group

h_{tot} = Total number of peptide bonds in a given protein (meqv/g protein)

The enzymatic reaction was terminated by heating the hydrolysate to 100 °C for 15 minutes. The cell debris was removed by centrifugation at 8,000 rpm for 15 minutes at 4 °C and pH was adjusted to 7.0 with 1 N HCl. The resulting silk hydrolysate was subjected to freeze-drying for overnight to obtain water-soluble silk powder, which was afterwards ground into fine powder.

Selection of commercial protease enzymes used for hydrolysis of silk pupae

The experiment was carried out following the previous procedure using Completely Randomize Design (CRD) and different commercial protease enzymes as 4 treatments, indicated as 1) control (without enzyme), 2) Alcalase 2.4L FG (Novozymes A/S, Denmark), 3) Neutrase 0.8L (Novozymes A/S, Denmark), and 4) Flavourzyme 500L (Novozymes A/S, Denmark) with the ratio of protein:enzyme = 6:0.5. Each experiment was performed in triplicate. The analysis was done using Analysis of Variance or ANOVA and Duncan's Mew Multiple Range Test (DMRT). The enzyme with highest %hydrolysis was selected as the most appropriate enzyme and used for further experiments.

Optimization for concentration of substrate, enzyme, and hydrolysis period for hydrolysis of silk pupae

The experiment was carried out at pH 9.5 and 60 °C using Central Composite Design (CCD) with 3 factors and 5 levels ($-\alpha$, -1, 0, 1, α). The first factor was substrate concentration varying from 4.6, 6, 8, 10, and 11.4%. The second factor was enzyme concentration ranging from 0, 0.2, 0.5, 0.8, and 1%. The last factor was hydrolysis period from 70, 90, 120, 150, and 170 minutes. Each experiment was performed in triplicate. The analysis was done using Response Surface Methodology (RSM) with

Quadratic Model. The condition with highest %nitrogen recovery[14] (NR) was selected as the most appropriate enzyme and was used for further experiments.

$$\text{nitrogen recovery (\%)} = \frac{\text{total nitrogen in hydrolysis x hydrolysate weight}}{\text{total nitrogen in raw material x raw material weight}} \times 100$$

Optimization for pH and temperature for hydrolysis of silk pupae

The experiment was carried out at optimal condition obtained from previous section using Central Composite Design (CCD) with 2 factors and 5 levels ($-\alpha$, -1, 0, 1, α). The first factor was reaction pH varying from 7.0, 7.5, 8.5, 9.5, and 10.0. The second factor was temperature ranging from 48, 50, 55, 60, and 62 °C. Each experiment was performed in triplicate. The analysis was done using Response Surface Methodology (RSM) with Quadratic Model. The condition with highest %nitrogen recovery (NR) and %hydrolysis was selected as the most appropriate enzyme.

RESULTS AND DICUSSION

Preparation and analysis of raw material

Analysis of silk pupa from *P. ricini* (Eri Silk) showed high moisture content as 74.66%, indicating liquid as its main component, protein 18.44%, lipid 4.24% and ash 1.58% as shown in Table 1. Comparison with *Bombyx mori* and *Attacus ricinii* pupae showed similarity as the main components were also moisture (65.13% and 70.14%, respectively) with protein and ash contents 11.99% and 0.79%, respectively for *B. mori* and 15.97% and 1.36%, respectively for *A. ricinii*[15]. However, differences were observed as the lipid content (20.10%) in *B. mori*, which was domestic strain, was much higher than those in *P. ricini* (4.24%) and *A. ricinii* (11.09%), which were both wild strains. The protein content, even though, was not as high as that in Eri silk cocoon,

96.32%, it still was rather high and then could be applied as protein sources for either cosmetics or foods.

Enzymatic hydrolysis of silk pupae

Selection of commercial protease enzymes used for hydrolysis of silk pupae

The protein hydrolysis reaction using enzyme as catalyst is very efficient, specific and can be carried out under mild conditions so that the nutritional quality of the amino acids is maintained[13]. For example, protein hydrolysis using alkaline solution not only destroyed some essential amino acids (tryptophane, cysteine or serine), but it also resulted in change of amino acid structure from *L*-form into *D*-form which human beings were not able to consume[16]. As a result, enzymatic catalysis is chosen for the experiments. Table 2 showed %hydrolysis results from hydrolysis with different protease enzymes. The hydrolysis level means the percentage of enzymatically hydrolyzed peptide bonds compared with the original peptide bonds in raw material representing a number of peptide bonds those being degraded during the reaction. In case that the level of hydrolysis is high, it indicates high level of protein hydrolysis into smaller peptides and free amino acids[13]. According to the results, it demonstrated that the highest %hydrolysis (52.39%) was obtained from Alcalase while the Neutrase and Flavourzyme gave 39.29% and 23.96% hydrolysis, respectively. Furthermore, the reaction without any protease enzymes at pH 7.0, 50 °C and at pH 9.5, 55 °C gave lower %hydrolysis (13.93% and 15.78%, respectively). Therefore, the protease enzyme was actually needed to enhance hydrolysis reaction.

Figure 1 showed the sizes of Eri pupa protein without and after the enzymatic hydrolysis. It indicated that the reaction without enzyme showed two prominent bands (82 and 40 kDa) which were similar to that before the hydrolysis reaction (data not

shown). In contrast, after hydrolysis, the prominent bands appeared not only at 82 kDa but also at 31 kDa with smear area between 7-16 kDa. This suggested that parts of pupa protein were hydrolyzed into smaller peptides by added enzymes. However, the hydrolysis patterns obtained from 3 commercial enzymes were rather different. The ones from Neutrase and Flavourzyme were very similar and gave hydrolysate with majority size around 82 and 31 kDa and minority size below 31 kDa whereas the one from Alcalase did not show the 82-kDa band at all but instead it demonstrated smear area below size 7 kDa. These results were consistent with %hydrolysis mentioned above in Table 2. Therefore, it could be concluded that the three protease enzymes possessed significantly different hydrolysis ability to pupa protein and Alcalase was the most efficient protease enzyme for hydrolysis of pupa protein with %hydrolysis = 52.39%. Thus, the Alcalase was selected for the next experiments.

Optimization for concentration of substrate, enzyme, and hydrolysis period for hydrolysis of silk pupae

Figure 2 showed protein sizes of Eri pupa hydrolysate from different hydrolysis reactions as monitored by 12% SDS-PAGE. The difference between reactions without and with enzyme was shown in lanes 2 and 3, respectively, that prominent bands around 75-100 kDa in lane 2 disappeared after enzymatic hydrolysis in lane 3. Furthermore, when the enzymatic reaction was prolonged from 60 to 120 minutes (lanes 3 and 4, respectively), the obvious protein bands around 10-25 kDa also disappeared indicating the effect of hydrolysis period. The effect of enzyme concentration on hydrolysis reaction was also demonstrated in lanes 5 and 6; the more enzyme used in the reaction, the more powerful protein hydrolysis was.

Figures 3 and 4 demonstrated the effects of substrate concentration, enzyme concentration and hydrolysis period on %hydrolysis and %nitrogen recovery, respectively, as shown by the Response Surface Curves. It was shown that the hydrolysis level and nitrogen recovery were slightly influenced by a period of hydrolysis (70-170 minutes) whereas they were strongly affected by the concentrations of substrates and enzymes. The hydrolysis level and percentage of nitrogen recovery tended to decrease as the substrate was more concentrated (4.6 – 11.4% protein) due to that the substrate was too concentrated than that the enzyme in range of experimented concentrations could be active so that the enzyme could not properly work and therefore resulting in poor hydrolysis reaction and nitrogen recovery. In contrast, when the concentration of enzyme was considered, it showed that the level of protein hydrolysis and nitrogen recovery were raised as more enzyme was added (0 - 1%). This indicated that more enzymes in the reaction could effectively bind to more substrates and then yielding more products. As also shown in the tannery fleshing protein hydrolysis reaction experimented by Raju et al. (1997)[17] that the enzyme concentrations played a very vital role in addition to other factors. In conclusion, the superimposed response surface curve between % hydrolysis and % nitrogen recovery (Figure 5) demonstrated that the most optimum hydrolysis condition for Eri pupa was 4.6% protein substrate, 0.5% enzyme and 120 minutes to obtain the highest nitrogen recovery 76.98%.

Optimization for pH and temperature for hydrolysis of silk pupae

Table 3 demonstrated the effect of pH and temperature of hydrolysis reaction on %hydrolysis and %nitrogen recovery. However, when the results were statistically analyzed using multiple regression in order to obtain the regression coefficients for the second order polynomial equation, the results indicated that the proposed model

possessed no significant ($p > 0.05$). It could be concluded that pH and temperature of hydrolysis reaction did not directly affect %hydrolysis and %nitrogen recovery. Therefore, the reaction with pH 7.5 and temperature 50 °C was selected as the optimal pH and temperature due to that this condition consumed the lowest energy.

Analysis of products

The Eri pupa hydrolysate powder contained 4.63% moisture, 11.47% total nitrogen, 1.70% lipid, 11.97% ash, total plate count, yeast and mold < 100 CFU/g with 100% water solubility. Comparison with the commercial products, "Promois®", demonstrated similarity in protein contents with less moisture and more ash contents as shown in Table 4.

Table 5 compared amounts of free and total amino acids in the products, it demonstrated that the sum of free amino acids was 16.40% out of 54.21% total amino acids with tyrosine, histidine, phenylalanine, leucine and arginine as main free amino acids (1.77%, 1.57%, 1.39%, 1.33% and 1.32%, respectively). This implied that enzymatic hydrolysis of pupa protein resulted in hydrolysate containing not only free amino acids but also water-soluble short-chain peptides. Furthermore, Table 6 showed types and amounts of amino acids present in the product compared with those in raw material and the results indicated similarity in amino acids profiles between the two samples.

CONCLUSION

Proximate analysis revealed that *P. ricini* (Eri Silk) pupa contained high protein as 18.44 % suitable for protein source in various applications. Eri pupa protein was also shown to be solubilized faster with commercial Alcalase 2.4 L FG, Neutrase 0.8 L and Flavourzyme 500 L than the reaction without protease enzyme. The recovery of soluble

nitrogen improved with increase in protease and substrate concentrations. The drgree of hydrolysis reached maximum to 79% to obtain most soluble nitrogen when hydrolysis condition was the best optimized. However, the condition with a slightly lower drgree of hydrolysis, 76%, was selected as the most appropriate condition for Eri pupa hydrolysate production due to the economic reason. Characterization of laboratory Eri pupa hydrolysate showed similarity in properties to the commercial silk hydrolysate. It showed greater content of total nitrogen with more percentage of ash and less moisture. Even though they were originally from different parts of silk, one from silk pupa and another from silk sericin, their properties could be comparable for substitution. Therefore, it is advantageous to consider scaling up the production process for industrial purpose, as well as incorporation such abundantly available inexpensive protein ingredients as one of substitutes to cosmetic or food industries in the future.

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Table 1 Chemical composition of silk worm

	% on fresh weight			
	Eri silk cocoon[1/]	Eri silk pupa[1/]	*Bombyx mori* pupa[2/]	*Attacus ricinii* pupa[2/]
moisture	4.26	74.66	65.13	70.14
ash	3.22	1.58	0.79	1.36
protein	96.32	18.44	11.99	15.97
lipid	1.55	4.24	20.10	11.09

[1/]Laboratory analysis

[2/]Mishra *et al.* (2003)

Table 2 Effect of different commercial protease enzymes on %hydrolysis

Tr.	Enzyme	Enzyme (%v/w)	Substrate (% protein)	Time (min)	pH	Temp (°C)	Hydrolysis (%)
1	No enzyme	0	6	90	7.0	50	13.93 d
2	No enzyme	0	6	90	9.5	55	15.78 d
3	Alcalase 2.4 L FG	0.5	6	90	9.5	55	52.39 a
4	Neutrase 0.8 L	0.5	6	90	7.0	50	39.29 b
5	Flavourzyme 500 L	0.5	6	90	7.0	50	23.96 c

Means in the same row with different letters are significantly different at 95% level ($p \leq 0.05$) as determined by Duncan's multiple range test

Table 3 Effect of different reaction pH and temperatures on %hydrolysis and %nitrogen recovery (under the condition %protein:enzyme = 4.6:0.5 for 120 minutes)

Tr.	Independent variance		Dependent variance	
	pH	Temperature (° C)	Hydrolysis (%)	Nitrogen recovery (%)
1	7.5	50	73.27	62.26
2	9.5	50	67.29	64.45
3	8.5	55	72.73	72.38
4	7.5	60	76.02	51.20
5	9.5	60	70.88	81.95
6	7.0	55	73.31	59.68
7	8.5	55	75.05	78.44
8	10.0	55	70.71	81.84
9	8.5	48	64.29	89.28
10	8.5	55	77.54	68.00
11	8.5	62	79.13	54.06

Table 4 Chemical and biological properties of products

Properties	Eri pupa hydrolysate powder	Commercial products "Promois®"	
		SILK-1000P[1/]	SERICIN-P[2/]
Moisture (%)	4.63	10.0	12.0
Total nitrogen (%)	11.47	13.0	13.0
Lipid (%)	1.62	not determined	not determined
Ash (%)	11.97	3.0	4.6
Total plate count (CFU/g)	< 100	not determined	not determined
Yeast and mold (CFU/g)	< 100	not determined	not determined

[1/] obtained from fibroin hydrolysis
[2/] obtained from sericin hydrolysis

Table 5 Comparison of free and total amino acids present in the product

Amino acids (g/100 g sample)	Eri pupa hydrolysate powder	
	Free amino acids	Total amino acids
Aspartic acid	0.76	5.23
Serine	1.27	4.02
Glutamic acid	1.20	6.77
Glycine	0.39	2.52
Histidine	1.57	2.47
Arginine	1.32	3.80
Threonine	0.86	3.45
Alanine	1.01	2.94
Proline	0.40	2.85
Cysteine	0.16	Not determined
Tyrosine	1.77	3.60
Valine	0.98	3.26
Methionine	0.57	Not determined
Lysine	1.04	3.83
Isoleucine	0.69	2.57
Leucine	1.33	3.82
Phenylalanine	1.39	3.08
Total	16.70	54.21

Table 6 Comparison of the products and raw material in terms of types and amounts of amino acids

Amino acids (%mol)	Eri pupa raw material	Eri pupa hydrolysate powder
Aspartic acid	8.51	9.29
Serine	6.82	9.04
Glutamic acid	10.90	10.95
Glycine	9.27	7.93
Histidine	3.16	3.76
Arginine	5.24	5.16
Threonine	4.23	6.84
Alanine	11.06	7.80
Proline	4.49	5.85
Cysteine	0.51	Not determined
Tyrosine	4.47	4.70
Valine	6.51	6.58
Methionine	2.31	Not determined
Lysine	6.35	6.19
Isoleucine	4.79	4.63
Leucine	7.70	6.88
Phenylalanine	3.69	4.41
Total	100	100

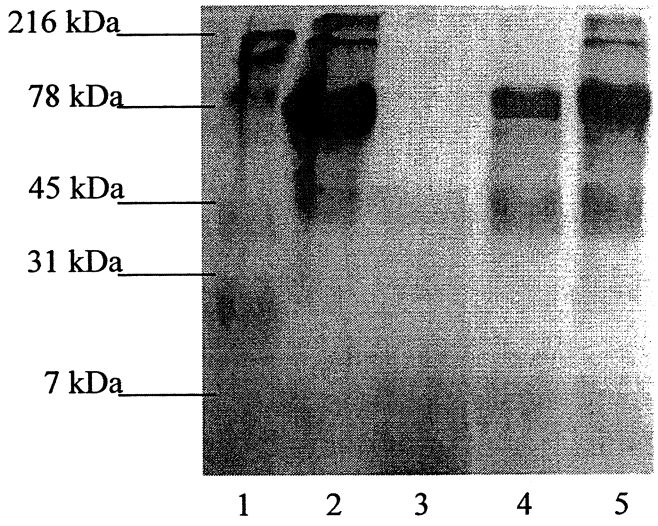

Figure 1 Protein size of Eri pupa hydrolysate as monitored by 12% SDS-PAGE. Lanes 1 and 6, protein molecular weight marker; (Kaleidoscope Prestained Standards, Bio-Rad, USA) which comprise myosin (216 kDa), BSA (78 kDa), carbonic anhydrase (45 kDa), soybean trypsin inhibitor (32 kDa) and aprotinin (7 kDa) Lane 2, Eri pupa hydrolysate hydrolyzed without enzyme; Lane 3, Eri pupa hydrolysate hydrolyzed with Alcalase; Lane 4, Eri pupa hydrolysate hydrolyzed with Nutrase; Lane 5, Eri pupa hydrolysate hydrolyzed with Flavozyme.

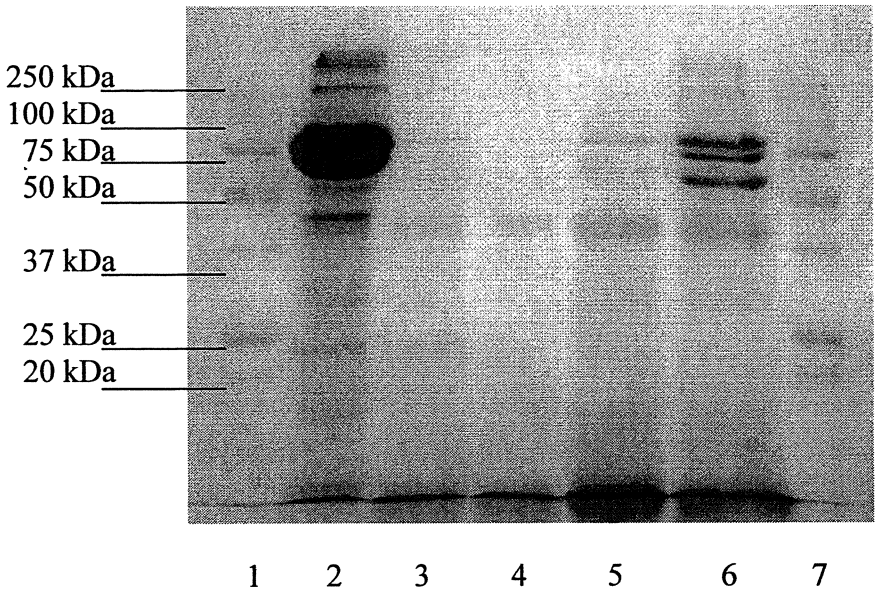

Figure 2 Protein size of Eri pupa hydrolysate as monitored by 12% SDS-PAGE. Lanes 1 and 7, protein molecular weight marker; (Precision Plus Protein™ Standards, Bio-Rad, USA) which contains 7 highly purified recombinant proteins in molecular masses from 20 to 250 kDa; Lane 2, Eri pupa protein:Alcalase 4.6:0 for 60 min; Lane 3, Eri pupa protein:Alcalase 4.6:0.5 for 60 min; Lane 4, Eri pupa protein:Alcalase 4.6:0.5 for 120 min; Lane 5, Eri pupa protein:Alcalase 10.0:1.0 for 120 min; Lane 6, Eri pupa protein:Alcalase 10.0:0.5 for 120 min.

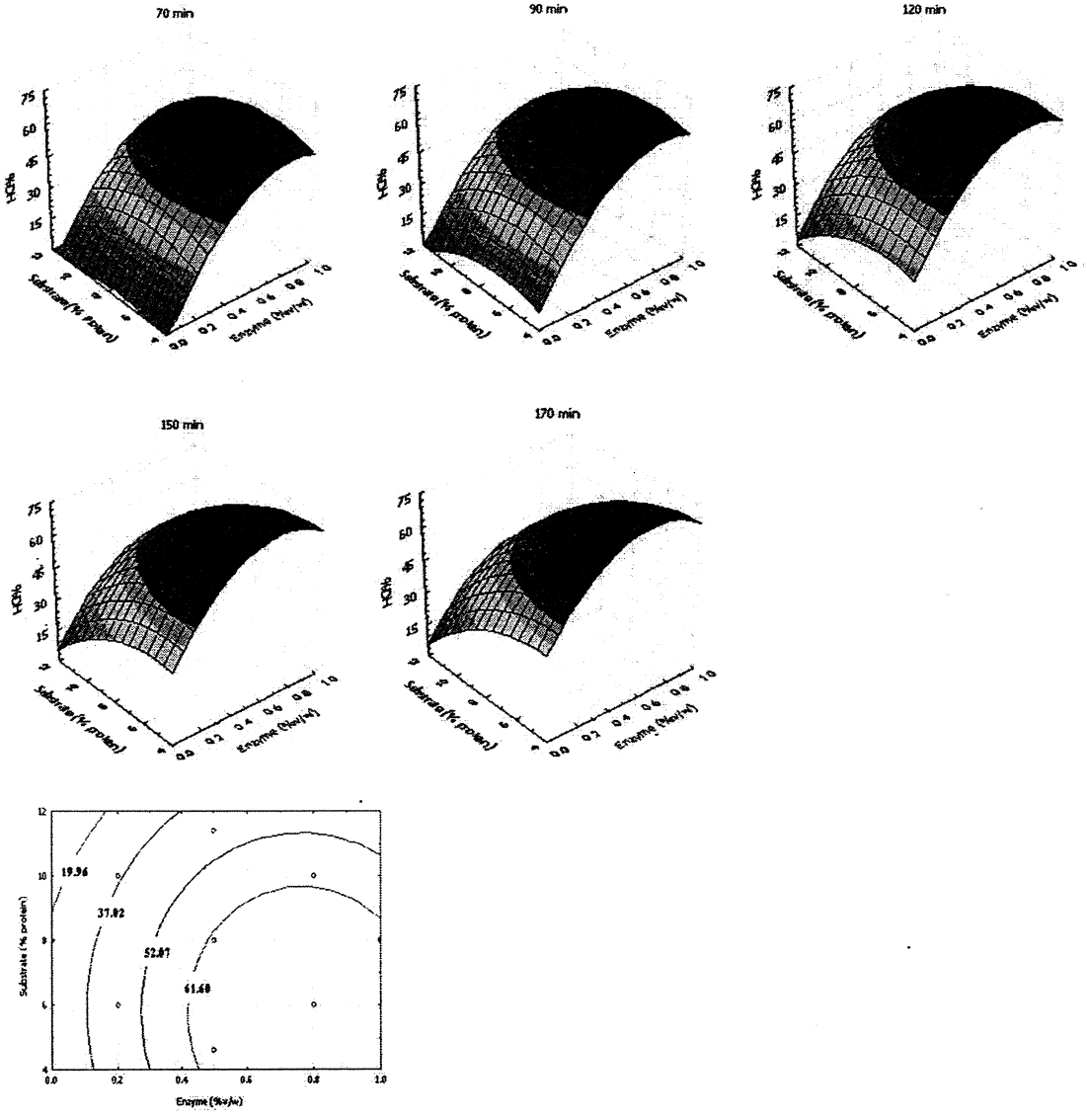

Figure 3 Effect of substrate concentration, enzyme concentration and hydrolysis period on % hydrolysis as shown by the Response Surface Curve

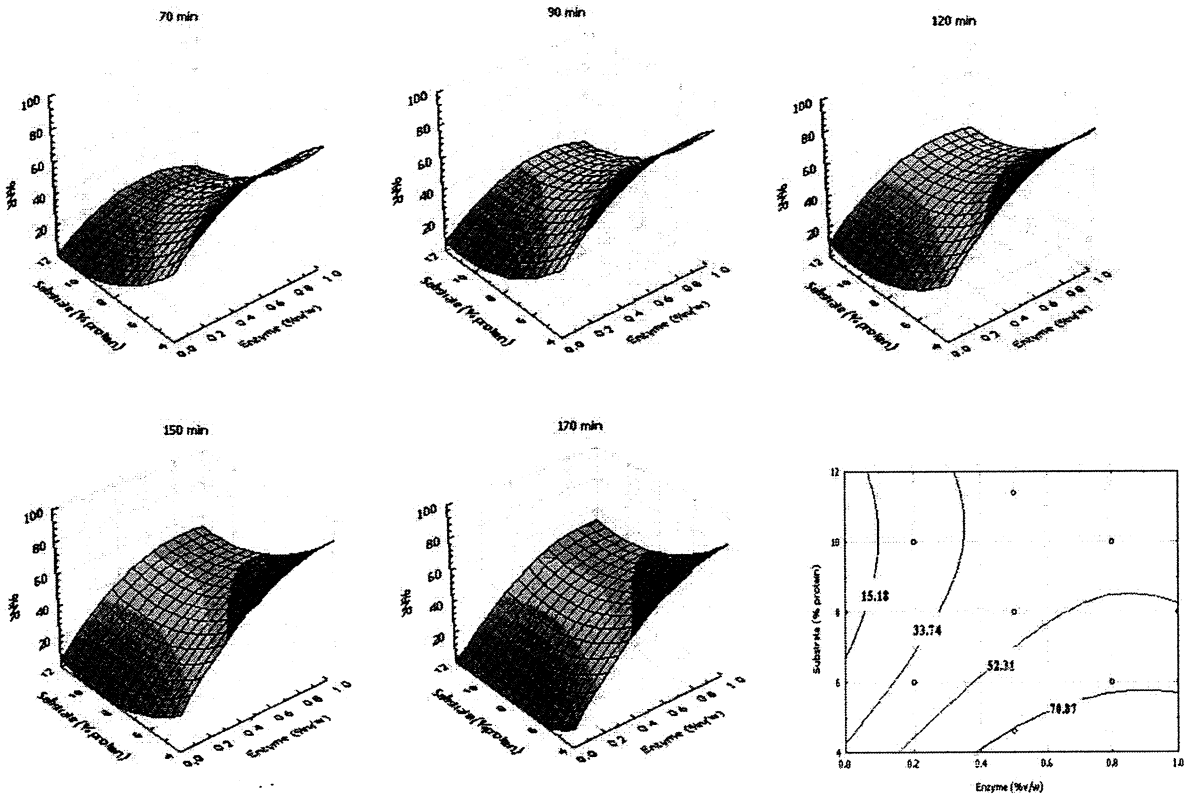

Figure 4 Effect of substrate concentration, enzyme concentration and hydrolysis period on %nitrogen recovery as shown by the Response Surface Curve

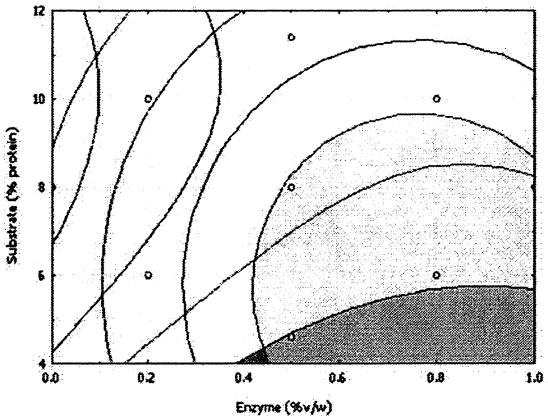

Figure 5 Effect of substrate concentration, enzyme concentration and hydrolysis period on %hydrolysis and %nitrogen recovery as shown by the Superimposed Response Surface Curve